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This proposal focuses on a novel topic that had not been investigated previously--i.e., potential derangements in expression or trafficking of scavenger receptors, LRP and megalin, and their interactions with uPA-R, uPA and PAI-1 in breast cancer cells. Upon beginning this work, our first order of business was to establish the systems required to carry out the proposed studies and to use them to evaluate the levels of expression of scavenger receptors in breast cancer vs normal cell lines. During the first year of the grant we have prepared several essential reagents (antibodies) and have characterized four cell lines --two estrogen-insensitive and one estrogen-sensitive breast cell lines and one normal mammary cell line. We have also demonstrated for the first time differences in the level of expression of scavenger receptors and the levels of expression and distribution of uPA-R, uPA and PAI-1 in normal vs tumor cells. The finding that the expression of uPA-R, uPA and PAI-1 is greater in estrogen-insensitive cells mimics the situation in vivo in breast carcinomas where overexpression of uPA-R and uPA is associated with tissue invasion and poor prognosis. The next step is to use the systems to investigate the interplay between scavenger receptors and uPA-R/uPA.

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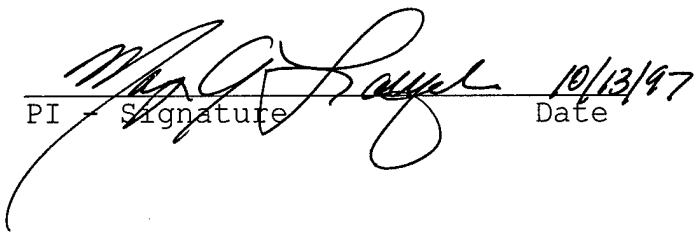

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PROGRESS REPORT

I. INTRODUCTION

Invasion and metastasis in breast cancer requires the action of tumor associated proteases which disrupt cell-cell and cell-substratum adhesion and facilitate cell motility. Most types of human adherent tumor cells and their corresponding normal counterparts use uPA to generate cell-bound plasmin which is a serine protease capable of degrading most extracellular proteins that are involved in the process of cell adhesion. Breast cancer cells are known to express higher levels of urokinase-type plasminogen activator (uPA), uPA receptor (uPA-R), and plasminogen activator inhibitor (PAI-1) than noncancerous mammary epithelial cells and high expression levels of these proteins is associated with poor patient prognosis. Yet, the mechanisms responsible for the accumulation of these components of the proteolytic cascade in such tumors are poorly understood. This is due to the fact that there are many gaps in our knowledge of how this proteolytic system is controlled. Specifically, little is known about the role of newly discovered scavenger or clearance receptors-- LDL receptor related protein/α2-macroglobulin receptor (LRP) and megalin--in the regulation of this proteolytic cascade.

The overall purpose of this research is to define the mechanisms responsible for the increased expression of uPA, uPA-R, and PAI-1 in breast cancers associated with increased potential for recurrence and metastasis. The working hypothesis we wish to test is that the delicate balance between protease activities and removal of the proteases from the extracellular environment is upset due, at least some cases, to alterations in the expression or trafficking of the newly discovered scavenger receptors, i.e., (LRP) and megalin. These receptors have recently been shown to be responsible for the uptake and clearance uPA:PAI-1 complexes, but little is known concerning their trafficking or how they interact with uPA-R. Among the possibilities that we wish to investigate that could explain why uPA, uPA-R and PAI-I accumulate in some cancer cells are as follows: 1) defects in either the sites or level of expression one of the two scavenger receptors, LRP or megalin, or 2) defects in the expression or trafficking of RAP (the specific intracellular chaperone for members of the LDL receptor family), or 3) failure to couple uPA-R to scavenger receptors for internalization and down-regulation of cell surface uPA binding sites.

We expected that the experiments proposed in the original application should shed light on a number of important questions including: 1) the comparative levels of expression of scavenger receptors in normal mammary epithelial cells vs breast cancer cells, 2) trafficking of scavenger receptors and their interactions with uPA-R in both normal and tumor cells; and 3) whether or not scavenger receptors are involved in the down-regulation of uPA-R expression through co-internalization. As a result, this study should provide new insights not only into the mechanisms responsible for the increased expression of uPA, its receptor and PAI-1 in breast cancer cells, but also on the normal trafficking and turnover of components of the uPA:uPA-R:PAI-1 system. Such new insights might be expected to have immediate application to the diagnosis/prognosis of breast cancer and would provide a rationale for designing novel therapeutic reagents.

The work proposed in the original application designed to gain insight into these problems was organized into four Specific Aims:

1. To determine the localization (by immunocytochemistry) and the levels of expression (by Western and Northern blotting) of the scavenger receptors and RAP in normal and tumor-derived mammary epithelial cell lines and human and mouse breast tumors. The goal of these studies is to define which of the two scavenger receptors is expressed in normal and tumor-derived mammary cell lines and in mammary tumors, whether the level of expression of these receptors or RAP is altered in mammary tumor-derived cell lines or

mammary carcinomas, and whether these receptors and RAP have the same intracellular and cell surface distribution in normal and tumor-derived mammary epithelial cells.

2. To compare the distribution and expression of uPA, uPA-R, PAI-1 and scavenger receptors in normal mammary epithelial cells vs. tumor-derived cell lines and mammary carcinomas with varying metastatic potential. A number of studies indicate that the amount of uPA and PAI-1 found in breast cancers with metastatic potential is greater than in normal breast tissue, but the comparative distribution of these proteins in mammary epithelial cells and mammary carcinomas and their relationship to scavenger receptors has not been established. Experiments will be carried out to answer the following questions: Does the distribution or level of expression of these proteins differ in normal and cancerous breast tissue? Are they expressed in the same or different cells? Are they located within the cell, at the cell surface or, in the case of intact tissue, within the extracellular matrix? Does the distribution of uPA-R and scavenger receptors coincide?

3. To determine the fate of uPA, uPA-R, scavenger receptors and uPA-PAI-1 complexes at the cell surface. uPA is normally bound to uPA-R at the cell surface, and when PAI-1 binds to uPA it triggers internalization of the complex via scavenger receptors. In these experiments we will quantitate after cell surface radioiodination the amounts of scavenger receptors, RAP, uPA and uPA-R found at the cell surface of normal and tumor-derived mammary epithelial cells. We will then bind ^{125}I-labeled uPA or uPA:PAI-1 complexes and RAP to the cell surface and study the fate of the ligands (uPA:PAI-1) and receptors (both uPA-R and scavenger receptors). The following questions will be answered: Are these proteins internalized? If so, are they taken up by scavenger receptors? Are they degraded in lysosomes, recycled to the cell surface, or partially degraded and proteolytic fragments released from the cell? Are uPA-R internalized? If so, are these receptors recycled or degraded? If these receptors are internalized, is internalization dependent on interaction with scavenger receptors?

4. To investigate the biosynthesis and fate of newly synthesized scavenger receptors, RAP, uPA-R, and PA1-1 in tumor-derived cell lines and mammary tumors propagated in nude mice. Our goals here are to establish the kinetics of intracellular processing and cell surface delivery of these proteins in normal vs tumor derived mammary cells. Our exact priorities will be dictated by results obtained in Specific Aims #1 and #2. These studies will allow us to determine whether the increased amounts of uPA, uPA-R and PAI-1 found in malignant breast tumors is due to increased synthesis, decreased degradation, or disturbances in the trafficking of scavenger receptors or RAP, and whether these proteins differ in some way (e.g., posttranslational processing) from their counterparts produced in normal mammary epithelial cells.

During the first year of the grant we have followed the work plan precisely as outlined in the original application. We have made considerable progress on Specific Aim #1 and Specific Aim #2, and we have begun work on Specific Aim #3. In addition, we have generated a number of new reagents including antibodies and cDNAs necessary to carry out this work. Details of our progress are spelled out below.

II. BODY: DETAILS OF PROGRESS

The hypothesis to be tested in this work is that the increased expression of uPA, uPA-R and PAI-1 associated with breast cancer cells is due to abnormalities in the expression or trafficking of one of the scavenger receptors (LRP or megalin) or RAP, a chaperone-like molecule that inhibits ligand binding to the receptors. Work that has been carried out to date under each specific aim is as follows:

1. SPECIFIC Aim #1: Determine the Sites and the Levels of Expression of the Scavenger Receptors and RAP in Normal and Tumor-derived Mammary Epithelial Cells

The goal here was to establish which cell lines and mammary tumors express LRP and megalin, and to determine the location and levels of expression of these proteins and RAP by immunocytochemical and immunochemical procedures. Our first order of business was to follow up our preliminary results and determine if the scavenger receptors and RAP are expressed in normal and tumor-derived human mammary epithelial cells and in human breast cancers using specific anti-RAP, anti-megalin, and anti-LRP antibodies.

Information Available from Previous work: Previous work had established that: a) epithelial cells of the normal human mammary epithelium (like the majority of cells in normal rat and human tissues) express RAP and one of the scavenger receptors (i.e., either LRP or megalin) (1, 2), and b) at steady state most (>90%) of the receptors are found at the cell surface, whereas most (>95%) of the RAP is found intracellularly (1, 3, 4) in the endoplasmic reticulum (ER) (3-5). Except for our preliminary studies, the expression of scavenger receptors had not been studied in normal or tumor-derived mammary cell lines or in mammary tumors. The only published information available was from a single study in which expression of LRP was found to be decreased 2-3-fold in MCF-10A mammary cells transfected with mutated Ha-ras (6).

Antibodies (See Table I): We had available upon beginning of this work a number of well characterized antibodies we had generated for our previous studies on scavenger receptors that were useful for immunocytochemical, immunoblotting and immunoprecipitation experiments (3, 4, 7-9). Using the resources of this grant we have generated two new polyclonal antibodies--one against human megalin and another against recombinant human uPA-R. We have also tested a number of commercially available anti-uPA-R monoclonal antibodies (MAbs) and have identified several that are useful for our purposes. A detailed list of the antibodies used in this study together with the assays for which each is useful is given in Table I. The precise antibody used in any given experiment is indicated in the appropriate figure legend.

a. Immunocytochemical Localization of Scavenger Receptors and RAP

1) Cell Lines: We have screened by immunofluorescence a number of normal and tumor-derived human mammary cell lines and selected four on which we have concentrated our efforts: An immortalized normal mammary cell line, 184-B5 (10), one estrogen-sensitive mammary carcinoma cell line MCF-7, and two estrogen-insensitive mammary carcinoma cell lines MDA-MB-231 and Hs578T. Note: We initially proposed to use the Hs578Bst cell line derived from myoepithelial cells as our normal cell line; however, we decided to switch to the 184-B5 cell line which is derived from ductal epithelium, as all the tumor cell lines are derived from infiltrating ductile carcinomas.

Methods: Methods were the same as those used in our recent studies of the distribution of megalin and RAP in various cell lines (3, 8, 9) and in rat kidney tissue (4, 11). In brief, for immunofluorescence cell lines are cultured on coverslips or in 8 well chambers, fixed in 2% paraformaldehyde in phosphate buffer or in paraformaldehyde-lysine-periodate (PLP) fixative for 1-3 hr, incubated with primary antibodies (1-2 hr) followed by FITC- or TRITC-conjugated secondary antibodies, and examined in a Zeiss Axiophot equipped for epifluorescence or by confocal laser scanning microscopy.

For immunoelectron microscopy cells are fixed in PLP or a mixture of 3% paraformaldehyde/0.5% glutaraldehyde, or 8% paraformaldehyde (30 min) followed by 4% paraformaldehyde in phosphate buffer for 4 hrs. They are then cryoprotected by infiltration (1 hr) with sucrose containing PVP, mounted on aluminum nails, frozen in liquid nitrogen, and ultrathin cryosections are cut at liquid nitrogen temperatures (12, 13) with a Reichert Ultracut ultramicrotome equipped with a cryoattachment, picked up onto nickel grids, and incubated with

primary antibody followed by incubation with goat anti-rabbit or anti-mouse IgG coupled to 5 or 10 nm gold particles. Details of these methods are given in recently published papers (3, 9, 14, 15). In some instances we also have used an immunoperoxidase labeling procedure in which cultured cells are fixed, reacted with primary antibodies followed by HRP-conjugated secondary antibodies, the DAB reaction carried out, and cells processed for routine EM (3, 4, 16). We have had considerable experience in carrying out immunofluorescence, immunoperoxidase and immunogold labeling on a variety of cultured cells and tissues over the last ten years and have written several reviews on this topic (17, 18).

Results (Summarized in Table II): Our data indicate that all cells--both normal and tumor cells--express LRP and RAP and that there are no notable differences in their distribution between normal and tumor cells. **LRP (Fig. 1):** To study the distribution of LRP we employed two different antibodies, one raised against whole LRP designated anti-LRP (human) and a second raised against a peptide whose sequence is from the cytoplasmic tail of LRP designated anti-LRP_{ct}. By immunofluorescence, both antibodies gave the same results; LRP was found both at the cell surface where it had a punctate distribution (suggesting its distribution in coated pits) and inside the cell concentrated in the Golgi region. Localization at the EM level (**Fig. 2**) verified that LRP is concentrated in its usual location at the cell surface--i.e., in clathrin-coated pits, and that intracellular receptors are found in Golgi cisternae and associated vesicles. By contrast, **megalin (Fig. 3)** was not detected in the 184-B5 normal mammary cell line by immunofluorescence, but was expressed in variable amounts in all tumor cell lines where it was found in its usual punctate distribution at the cell surface in clathrin-coated pits. The highest level of expression was found in Hs578T > MDA-MB-231 > MCF-7 cells. Interestingly, in Hs578T cells megalin was often seen at the leading edge of migrating cells (Fig. 3D). **RAP (Fig. 4)** was found in all cell lines--both normal and tumor derived--and was concentrated in the ER where it overlapped with the ER marker, protein disulfide isomerase (PDI).

Conclusions: All cell lines tested were found to express LRP and RAP. No differences in subcellular distribution were detected between normal and tumor cell lines. Curiously, megalin was expressed in the tumor cell lines but not in the normal cell line and the level of expression was greatest in the estrogen-insensitive cell lines where megalin was frequently seen at the leading edge of migrating cells. We are in the process of testing more normal mammary cell lines to see if this is a general observation that applies to other estrogen-insensitive cell lines.

2) *In Vivo* Breast Cancer Models: We have carried out preliminary experiments by immunofluorescence on the localization of LRP, megalin, and RAP in tumors composed of MDA-MB-231 cells grown in nude mice. Tumors are grown by injecting $1x10^7$ cells into the 3rd mammary gland of female nude mice. The tumors are allowed to grow to 5-10 mm in diameter after which the animals are euthanized, and the tumor tissue is removed and fixed for immunocytochemistry.

Methods: Tissue is fixed in PLP or 8% paraformaldehyde (30 min) followed by 4% paraformaldehyde in phosphate buffer for 4 hrs. It is then cryoprotected as described above and semithin (0.5-1 μM) cryosections are cut at liquid nitrogen temperatures as described above for ultrathin cryosections. Sections are transferred to slides for antibody incubations as described above for immunofluorescence of cultured cells.

It is of particular interest to us to check the expression of scavenger receptors in tumors produced from MDA-MB-231 cells to determine whether there is a difference between these cells in culture and those grown in vivo in nude mice.

Results (Fig. 5A): In preliminary experiments we found that LRP is weakly expressed in tumors derived from MDA-MB-231 cells expressed in nude mice. Further experiments are now in progress.

3) Normal Human Breast Tissue and Breast Tumors: We have examined 28 cases of primary breast carcinomas of varying states of differentiation obtained from surgical specimens removed from patients at the time of mammoplasty or removal of breast cancers. Sections were prepared in the tissue core of the UCSD cancer center and screened by immunoperoxidase for expression of LRP, megalin and RAP. Tissue was provided by Dr. Nissi Varki, Director of the UCSD Cancer Center Tissue Core or by Dr. Dontscho Kerjaschki, Professor and Chairman, Department of Pathology, University of Vienna. The distribution of these proteins in normal breast tissue at the margins of the tumors were compared to those in the tumors.

Methods: Tissue sections, 5 μm thick, are cut from representative blocks of tissue that have been fixed in 10% phosphate-buffered formaldehyde (pH 7.4) and embedded in paraffin. Sections are mounted on slides, deparaffinized in zylene and used for immunohistology using the Vectorstain ABC kit for immunoperoxidase.

Results (Fig. 5B-D): LRP and RAP were widely distributed in epithelial cells as well as in fibroblasts, macrophages and mast cells found at the margins of the tissues sections in mammary tumors as well as in normal breast tissue; however, there was variability from one field to another. In general the reaction was strong in fibroblasts and macrophages and highly variable in carcinoma cells. **Megalin** was not detected in normal breast tissue or in mammary tumors using anti-megalin$_{ct}$. The next step is to check this result using our new antibody raised against human megalin which, as expected, appears to be more sensitive for the detection of megalin in human tissues than antibodies raised against rat megalin.

Conclusions: By immunoperoxidase LRP and RAP are expressed in both normal breast tissue and in breast cancer where it is found in several cell types--normal epithelial cells and carcinoma cells, fibroblasts and macrophages. Megalin was not detected in tumors or normal tissue with the anti-megalin$_{ct}$, but this result needs to be checked with our new, more sensitive human anti-megalin antibodies.

b. Western Blotting

The expression levels of LRP, megalin and RAP were evaluated and compared by immunoblotting (LRP and RAP) or immunoprecipitation (megalin) in the same four mammary cell lines as described above by methods used previously (4, 7, 8).

Methods: For immunoblotting, cells are grown to confluency on 100-mm culture dishes and proteins are extracted in detergent (10 mM CHAPS). Equal amounts of detergent extracted proteins from each cell line are separated by SDS-PAGE on 5-10% gradient gels, transferred to poly(vinylidene difluoride) membranes using a Biorad wet-tank transfer apparatus, and incubated with specific anti-LRP, anti-megalin, or anti-RAP antibodies. Bound antibodies are detected by chemiluminescence (ECL kit) which provides us with a semi-quantitative assessment of protein expression. For immunoprecipitation, confluent cultures of cells are radiolabeled by incubating with [^{35}S]methionine/cysteine (1 mCi/100 mm dish), for 18 hrs, lysed in detergent as described above, and immunoprecipitates prepared by incubating cell lysates with anti-megalin antibodies and protein A immobilized on agarose beads. Antibody bound proteins are solubilized with Laemmli sample buffer containing 4% β-mercaptoethanol, and resolved by SDS-PAGE.

Results (Summarized in Table II): All four cell lines expressed **RAP (Fig. 6B)**, and the amount of RAP was similar in the three tumor cell lines and the normal cell line. By contrast, the levels of **LRP (Fig. 6A)** varied significantly among the four cell lines with MDA-MB-231 >>> Hs578T >> 184-B5 >>> MCF-7 cells. So far studies with megalin are more limited as antibodies against human megalin have only recently been generated, but by immunoprecipitation (IP), **megalin (Fig. 6C)** could be detected in Hs578T cells which is in agreement with our immunofluorescence data. Studies of the remaining cell lines are now in progress.

Conclusions: The immunochemical and immunocytochemical results demonstrate that normal and tumor cells express comparable amounts of RAP, that LRP expression varies considerably, and that megalin (assessed so far mainly by immunofluorescence (IF)) is expressed in all tumor cells but not in a normal mammary cell line. Interestingly, the expression

levels of LRP when compared between the tumor cell lines directly correlate with tumorigenicity in nude mice with MDA-MB-231 > Hs578T > MCF-7 cells. The expression of megalin as detected by IF also correlates with malignancy in that it is found in all tumor cells but not in a normal mammary cell line.

Summary of Results and Questions Answered: The results of these studies have established: 1) which cell lines express LRP, megalin and RAP and where in the cells these proteins are located; 2) that there are differences between a normal mammary epithelial cell line and estrogen-sensitive and estrogen-insensitive tumor-derived cells in the levels of expression of scavenger receptors but not RAP. Particularly interesting is the finding that the level of expression of scavenger receptors is highest in estrogen-insensitive tumor cells and that megalin is expressed in tumor cell lines but not in normal mammary cells. Our overall goal is to determine whether there are derangements between normal and tumor cells in the expression or location of scavenger receptors that might be responsible for the altered levels of uPA, uPA-R, and PAI-1. Our findings to date indicate that the altered levels of the latter cannot be explained by the failure of scavenger receptors to be expressed in tumor cell lines. Thus we must consider other possibilities--e.g., decreased expression of scavenger receptors at the cell surface or their mislocalization to different domains of the plasma membrane which would be expected to result in decreased clearance of uPA, uPA-R, and PAI-1 and their accumulation at the cell surface. For now we can say that there are variations between normal and tumor cells in the level of expression of scavenger receptors, and we must delve deeper through the studies planned under Specific Aims #2-4 to understand the mechanisms involved.

2. SPECIFIC AIM #2 (Year 1): Compare the Distribution and Expression of uPA, uPA-R, PAI-1, and Scavenger Receptors in Normal Mammary Epithelial Cells and in Mammary Carcinomas with Varying Metastatic Potential

Information Available from Previous Work: A number of recent studies have reported that the levels of expression of uPA, uPA-R and PAI-1 are all higher in breast cancers with metastatic potential than in normal breast tissue or carcinoma-in-situ (19-24). Information on the distribution of these proteins in normal mammary epithelial cells and mammary tumors is limited to a few light microscopic studies. By immunohistochemistry uPA (22, 25), uPA-R (26-28) and PAI-1 (22, 23, 25) were detected both at the cell surface and in the cytoplasm of mammary tumor cells . PAI-1 was also detected in the extracellular matrix (basement membrane) (23). In some cell lines uPA-R has been detected in focal adhesions, but in MCF-7 mammary carcinoma cells and colon carcinoma cells uPA-R has been localized to the dorsal ("apical") or nonadherent cell surface facing away from the culture dish and to the leading edge of migrating cells (29). This suggests that the distribution of uPA-R is altered in carcinoma cells. None of the components of the uPA-R:uPA:PAI-1 system has been localized in breast cancer cells at the electron microscope level up to now.

The goal of these studies is to find out where in or on cells uPA, uPA-R, and PAI-1 are located, if they are found on the same or different cells, and, if on the same cells, whether they colocalize in the same organelles, especially whether they are found in the same domains and microdomains of the cell membrane.

a. Localization of uPA, uPA-R, and PAI-1

We have begun to study the distribution of uPA, uPA-R and PAI-1 by immunofluorescence and Western Blotting in the same four cell lines used to study scavenger receptors and RAP as reported above. To carry out immunofluorescence studies we have obtained antibodies that recognize human PAI-1, uPA and uPA-R commercially (from American Diagnostics, Greenwich CT) (See Table I). These antibodies have been used in a number of

immunofluorescence and immunoperoxidase localizations at the light microscope level (29). We also tested a monoclonal antibody to uPA-R obtained from Dr. Mark Shuman, UCSF, but it was not useful for immunocytochemistry. In addition, we made a polyclonal antibody against recombinant human uPAR which is suitable for immunoblotting.

1) Results obtained with uPA-R antibodies (Summarized in Table III): We found that the distribution of uPA-R in the various cell lines differed depending on the antibody used. With one MAb (3936) which recognizes unoccupied but not occupied receptors, a punctate staining of the entire plasma membrane was observed in all tumor-derived cell lines **(Fig. 7)**. The only difference in uPA-R expression noted between the cell lines was in the number of individual cells in which expression was seen: uPA-R was detected in >80% of the two estrogen-insensitive cell lines (MDA-MB-231 and Hs578T), whereas only <40% of the MCF-7 (estrogen-sensitive) or the normal cell line showed such plasma membrane staining. With another MAb (3937) which detects both occupied and unoccupied receptors, uPA-R was found in the same distribution as with MAb 3936, but a major difference was evident: In MDA-MB-231 and Hs578T cells, staining was seen at focal adhesions on the basal (facing the dish) surface of the cells. With our polyclonal antibody, uPA-R was detected at the plasma membrane but was also found in intracellular compartments, particularly in the ER, suggesting that this antibody, which was made against a denatured recombinant protein expressed in bacteria, recognizes precursor forms of uPA-R whereas the MAbs recognize only the mature, cell surface receptors.

Conclusions: These results demonstrate that uPA-R can be detected with our reagents in all tumor cell lines and in the normal mammary cell line by immunocytochemical methods. They also suggest that occupied receptors, at least in some cases, may have a different distribution than unoccupied receptors in that the former were found to be concentrated at focal adhesions and the latter along the entire plasma membrane in both MDA-MB-231 and Hs578T estrogen-insensitive cell lines. The concentration of occupied receptors at focal adhesions in one of the most malignant cell lines has important functional implications. Focal adhesions are sites of concentration of integrin receptors and signaling molecules which are known to regulate cell attachment. The concentration of occupied uPA-R at focal adhesions in more agressive cell lines suggests that these receptors with their bound proteinase might influence cell attachment. Thus this result deserves to be followed up and extended to other tumor cell lines.

2) Results obtained with uPA and PAI-1 antibodies (Summarized in Table III): These studies are still in their infancy, but what we have found so far is intriguing: In MDA-MB-231 and Hs578T cells, uPA was distributed in a punctate staining pattern along the entire apical cell membrane as well as in presumptive focal adhesions at the basal cell surface (**Fig. 8**). PAI-1 was found associated with fibrillar extracellular matrix-like material at the basal surface of these cells. By contrast, in the normal mammary cell line, 184-B5, uPA was present only on the apical cell surface and was not detected in focal adhesions, and PA-1 was not detected in the extracellular matrix-like material. MCF-7 cells were negative for both uPA and PAI-1.

Conclusions: While tentative, these results suggest that uPA is concentrated at focal adhesions in the estrogen-insensitive breast cancer cell lines but not in a normal mammary cell line. Moreover, PAI-1 was not detected in the latter but was abundantly expressed by the tumor cells where it is deposited in the extracellular matrix. These results also raise the question of whether uPA, PAI-1 and uPA-R are bound together in a complex or are in close proximity to one another. To answer this question will require combined immunoelectron microscopy and immunoprecipitation studies which are now in progress.

b. Confirmation of Expression by Western Blotting

The expression of uPA, PAI-1 and uPA-R was also checked by immunoblotting of cell extracts prepared from each of the four cell lines selected for study.

Results (Summarized in Table III): Immunoblotting for uPA-R was carried out on cell lysates prepared by detergent extraction as described above for scavenger receptors and RAP. The results confirm that both the normal and all tumor cell lines express uPA-R (**Fig. 9**). Semi-quantitative analysis suggested that there is no significant difference in the expression levels of uPA-R between the tumor cell lines; however, all breast cancer cell lines express more uPA-R than does the normal mammary cell line. Immunochemical quantitation of uPA and PAI-1 expression is currently in progress.

Summary of Results and Questions Answered: In the original proposal we put forward a number of questions which would be answered by the above experiments: Does the distribution of uPA-R, uPA, or PAI-1 differ in normal and cancerous breast tissue? Are they expressed in the same or different cells? Are they located within the cell, at the cell surface or, in the case of intact tissue, within the extracellular matrix? If within the cell, does their distribution overlap with ER, Golgi or lysosomal markers? What are the expression levels of these proteins in normal and tumor-derived mammary epithelial cells? Are the components of the uPA-R:uPA:PAI-1 system colocalized in the same intracellular organelles and in the same regions of the plasma membrane--i.e., leading edge, apical or basolateral (facing the dish) cell surfaces?

We have obtained at least tentative answers to many of these questions. We have found 1) that uPA-R is located on the cell surface and within intracellular compartments (ER) in both estrogen-sensitive and insensitive breast cancer cell lines as well as in the normal mammary cell line; 2) that the levels of uPA-R, uPA and PAI-1 expression are increased in tumor cell lines which directly correlates with the situation in breast cancers in situ; 3) that PAI-1 is found predominantly in the extracellular matrix deposited by the breast cancer cell lines but is absent from that of the normal cell line; 4) that the distribution of uPA differs between normal (apical surface only) and tumor cell lines (apical surface plus focal adhesions). In the case of uPA-R we have also obtained suggestive evidence that unoccupied and occupied receptors have a different distribution in some cell types with the occupied receptors concentrated at focal adhesions. The next step is to move forward from these qualitative (immunocytochemical) and semi-quantitative (Western blotting and ECL detection) approaches to accurately quantitate their expression levels at the cell surface and to precisely localize these proteins at the EM level.

3. SPECIFIC AIM #3 (Years 2 and 3): Determine the Fate of uPA, uPA-R and uPA: PAI-1 Complexes at the Cell Surface

Information Available from Previous Work: It has been established by the work of others that normally uPA is bound to uPA-R at the cell surface, and when PAI-1 binds to uPA it inactivates the enzyme and triggers internalization of the uPA-R:uPA:PAI-1 complex (1) via scavenger receptors (30-33). However, in breast cancers with metastatic potential there is increased expression of uPA, uPA-R and PAI-1. This suggests that there could be some abnormality in the binding and/or clearance of the complexes via scavenger receptors in breast cancer cells. The studies carried out under Specific Aims #1 and 2 have established, as we predicted, that there are abnormalities in the distribution and expression of uPA-R, and the studies in this specific aim are designed to establish whether there are abnormalities in their clearance function (i.e., internalization and endocytic trafficking). Before doing so we must accurately determine if the quantity of scavenger receptors is comparable between normal cells and tumor-derived cells and measure the number of functional binding sites for uPA:PAI-1 on each cell type. This measurement will allow us to determine if the receptor-specific ligand binding activity on tumor cells is altered compared to normal cells.

a. Quantitation of uPA-R and Scavenger Receptors at the Cell Surface

Since uPA-R and scavenger receptors are expressed on both normal and tumor-derived cell lines it became essential for us to quantitate the amount of scavenger receptors and

uPA-R found at the cell surface of normal and tumor-derived mammary epithelial cells and to determine if they function normally.

1) Assessment of Cell Surface Expression by Immunoprecipitation: The goal of these experiments is to compare the amounts of scavenger receptors and uPA-R expressed at the cell surface between the breast cancer cell lines by immunoprecipitation following cell surface radioiodination.

Methods: Normal mammary epithelial cells and breast cancer cell lines are radioiodinated by lactoperoxidase-mediated cell surface iodination as previously described (8). Cells are then lysed in detergent and radiolabeled proteins are immunoprecipitated using anti-megalin, anti-LRP and anti-uPA-R antibodies. The expression levels of scavenger receptors and uPA-R are quantitated by analysis on a Molecular Dynamics Phosphorimager using ImageQuant version 3.3 software.

Results obtained for LRP (Fig. 10): Our results demonstrate that the number of receptors expressed on the two most malignant, estrogen-insensitive cell lines (MDA-MB-231 and Hs578T) is much greater than that which is detected on the estrogen-sensitive cell line (MCF-7) with MDA-MB-231>>Hs578T>>>MCF-7. These results are consistent with our semi-quantitative analysis of LRP expression using immunoblotting and chemiluminescence detection.

Results obtained for uPA-R: These experiments have been initiated only recently using our new uPA-R antibody, and we have run into a technical problem--that is the solubility of uPA-R in detergents. Proteins such as uPA-R with a glycosylphosphotidylinositol (GPI) lipid anchor tend to aggregate and precipitate when placed in detergents and as a result are not resolved by SDS-PAGE. At present we are in the process of testing different detergents (e.g. Triton X-100, β-D-octylglucoside, and SDS-containing buffers) in the extraction step for their ability to solubilize uPA-R for biochemical analysis. We will also try adding iodoacetamide to prevent the reformation of disulfide bonds following treatment with reducing agents.

Results obtained for megalin: We are currently in the process of testing our newly generated antibody.

2) Assessment of Functionality of Cell Surface Receptors: To obtain this data we need to determine if the scavenger receptors and uPA-R are able to bind their respective ligands with high affinity and internalize the bound ligand by receptor-mediated endocytosis. We are in the process of preparing the N-terminal fragment of uPA as a recombinant fusion protein which will contain the binding site for uPA-R in order to quantitate the number of functional binding sites that are present on each cell type for this receptor. α2-macroglobulin (α2M) represents a specific ligand for LRP that is not taken up by megalin or any other receptor and therefore is ideal for assessing the functionality of LRP. Finding a specific ligand for megalin constitutes a problem as all of the ligands taken up by this receptor are also taken up by LRP rendering it difficult to distinguish between internalization by megalin vs LRP. The only preliminary results obtained so far are with LRP.

Methods: To determine the relative number of functional binding sites on the cell surface, ligands are radioiodinated as done previously for RAP-GST (7, 8) and bound to normal and breast cancer cell lines at 4°C. After removing unbound ^{125}I-ligands, cell associated radioactivity is determined by gamma counting. The bound ligand is then quantitated by dividing the counts/min (cpm) by the specific activity of the radiolabeled ligand (cpm/ng protein). The amount of specifically bound ligand is then normalized to total cellular protein to permit a direct comparison between cell lines. To determine ligand/receptor binding affinities, cells are incubated at 4°C with a predetermined concentration of radiolabeled ligand in the presence of increasing amounts of unlabeled ligand. The concentration of unlabeled ligand that results in 50% inhibition of ^{125}I-ligand binding to cells represents the binding affinity (Kd). To measure receptor-mediated ligand internalization, cells are incubated with ^{125}I-ligands at 37°C, and the media sampled at various times, adjusted to 10% trichloroacetic acid (TCA), and non-precipitable material measured by gamma counting. The TCA soluble material is a direct measurement of intracellular (lysosomal)

degradation of the ligands over time and is a well established procedure for quantitating receptor-mediated endocytosis.

Results obtained for LRP (Figs. 11-13): The relative amount of functional LRP on the cell surface varied greatly between the cell types. MDA-MB-231 cells have almost 10X more receptors on their surface than the other two breast cancer cell lines (Hs578T and MCF-7) and the normal mammary cell line (184-B5) (Fig. 11). In addition, we found that all three breast cancer cell lines demonstrated a slightly lower affinity for α2M binding to LRP (Kd=0.45 nM) than the normal cells (Kd=0.14 nM) (Fig. 12). When the rate of uptake and degradation of ^{125}I-α2M was measured, we found that the normal mammary cells and Hs578T cells degraded the ligand in a linear, time-dependent manner indicating that LRP is constitutively recycled for successive rounds of ligand uptake (Fig. 13). MCF-7 cells showed little degradation of ^{125}I-α2M which is consistent with their low level of LRP expression. The most intriguing results were those obtained with MDA-MB-231 cells which exhibited rapid uptake and degradation of ^{125}I-α2M (indicating rapid internalization and degradation of bound ligand) followed by very little degradation over the remaining 6 hour time course. These results suggest that in MDA-MB-231 cells, LRP undergoes one rapid round of internalization to deliver its bound ligand to lysosomes and is then incapable of recycling to the plasma membrane for additional rounds of ligand uptake.

Conclusions: The amount of functional LRP expressed on the surface of each cell type as determined by quantitating ^{125}I-α2M binding closely correlates with the quantitative data we obtained for LRP expression by immunoblotting and immunoprecipitation with MDA-MB-231 cells expressing much greater amounts (X10) of LRP than other cell lines. Thus there is no detectable difference in α2M binding affinities for the receptor in tumor-derived cells. However, ligand binding is only the first step in scavenger receptor cycling. Binding is normally followed by internalization of the receptor/ligand complex with delivery of the ligand to lysosomes for degradation and recycling of the receptor to the plasma membrane (34). Our results suggest that receptor recycling takes place with normal kinetics in all except MDA-MB-231 cells which show a defect in receptor recycling. This is indicated by the finding that although MDA-MB-231 cells express large numbers of functional LRP on the surface there is a decreased rate of ligand degradation in these cells. This finding is intriguing and needs to be followed up because it suggests that at least some of the most malignant breast cancer cells show defects in scavenger receptor function.

4. SPECIFIC AIM #4 (Years 2 and 3): Investigate the Biosynthesis and Fate of Scavenger Receptors, RAP, uPA-R, and PA1-1 in Tumor-derived Cell lines and Mouse Tumors

No progress to report yet. The studies outlined under this Specific Aim have not yet been initiated.

IV. OVERALL CONCLUSIONS

General Statement: In the first year of our proposal we have made significant progress on Specific Aims #1 and #2 and have started experiments outlined under Specific Aim #3. We have also made progress on each of the tasks outlined in the Statement of Work for the first year and have moved ahead of the original schedule in some cases (e.g., quantitative analysis of scavenger receptors). We have concentrated on characterizing the expression levels and subcellular distribution of the scavenger receptors (LRP and megalin), uPA-R, uPA, and PAI-1 in 3 breast cancer cell lines (MDA-MB-231, Hs578T, and MCF-7 cells) and 1 normal mammary epithelial cell line (184-B5). The most important general conclusion we can make at this point is that we have documented a number of differences between the cell lines studied in either expression levels of these proteins or their localization which lend credence to our original working hypothesis for the entire grant--i.e., that scavenger receptors may be abnormal in breast cancer cells and that they are the missing link that needs to be considered and analyzed in order to understand the connection between malignancy and expression of uPA-R, uPA and PAI-1.

Highlights of our results and conclusions to date are as follows.

1. The scavenger receptors, LRP and megalin, are expressed in all tumor cell lines investigated, and, in the case of LRP, these receptors function normally in ligand binding. Curiously, megalin is expressed in all 3 breast cancer cell lines but is not detected in the normal cell line. We need to investigate more normal and tumor-derived cell lines to determine if megalin expression is connected with the cancerous phenotype or whether this represents variation between different cell lines.

2. There is a direct correlation between the level of LRP expression in breast cancer cell lines and tumorigenicity in nude mice in the cell lines studied so far. Quantitative analysis of LRP expression levels in the most malignant cell line studied, MDA-MB-231 cells, by immunoblotting, immunoprecipitation, and ligand binding demonstrates that they express 10X greater levels of LRP than non tumor-producing breast cancer cells (MCF-7) or a normal mammary cell line (184-B5). Ligand binding and uptake studies suggest that while ligand binding is normal in these cells, receptor recycling is impaired in this cell type. This is a very intriguing finding which we now need to expand to other tumorigenic cell lines.

3. RAP is expressed at similar levels in both normal and tumor cell lines and is found in its usual location in the ER. Thus the distribution and expression of RAP are similar to other cell types of varied origin studied previously. From these data it is safe to conclude that the differences in the expression of scavenger receptors cannot be explained by differences in the level of expression of their chaperone, RAP.

4. The levels of expression of uPA-R at the cell surface are considerably higher in the two more agressive (estrogen-insensitive) breast cancer cell lines as compared to the estrogen-sensitive and normal cell line. This matches the finding that expression of uPA-R is higher in more malignant breast tumors with increased potential for metastasis and indicates that the cultured cell lines provide a valid *in vitro* model to study the role of uPA-R in breast cancer. Moreover, our studies to date suggest that occupied uPA-R may have a different distribution in the more malignant cell lines. Unoccupied uPA-R are seen along the entire cell surface in all cell lines, but in MDA-MB-231 and Hs578T cells, occupied uPA-R were also associated with focal adhesion sites on the basal cell surface. This suggests that uPA-R could play a significant role in localizing uPA proteolytic activity near focal contacts and thereby promote cell detachment and migration. We need to verify this finding by colocalization of uPA-R at the EM level with uPA and focal adhesion markers such as integrin receptors, vinculin, paxillin and focal adhesion kinase

5. The protease, uPA, is found at the cell surface in all four cell lines However, in the two estrogen-insensitive cell lines but not in normal cells, uPA is also found at focal adhesion sites. These results suggest that increased uPA proteolytic activity may exist at sites of cell adhesion which would greatly enhance cell detachment and migration of breast cancer cells as compared to normal cells. This is a very intriguing finding and we will follow this up with closer examination of the distribution of uPA in breast cancer cells vs normal mammary cells during the coming year.

6. PAI-1 is a secreted protein that is known to associate with extracellular matrix (ECM) proteins. Our immunocytochemical results to date show that 1) PAI-1 is expressed in both estrogen insensitive breast cancer cell lines and is deposited in the ECM, and 2) it is not found in the ECM of the estrogen sensitive or normal cells. These results suggest that the expression of PAI-1 may directly correlate with the invasive phenotype of the most agressive breast cancer cell lines. We will perform quantitative studies to determine if any PAI-1 is being made by 184-B5 cells and other normal mammary cell lines and further evaluate PAI-1 expression levels in the breast cancer cells.

Bibliography

1. Zheng, G., D. R. Bachinsky, I. Stamenkovic, D. I. Strickland, D. Brown, G. Andres and R. T. McCluskey. 1994. Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, Gp330 and LRP/α_2MR, and the receptor-associated protein, (RAP). *J. Histochem. Cytochem.* 42:531-542.
2. Kounnas, M. Z., S. Stefansson, E. Loukinova, K. M. Argraves, D. K. Strickland and W. S. Argraves. 1994. An overview of the structure and function of glycoprotein 330, a receptor related to the α_2-macroglobulin receptor. *Ann. N.Y. Acad. Sci.* 737:114-123.
3. Lundstrom, M., R. A. Orlando, M. S. Saedi, L. Woodward, H. Kurihara and M. G. Farquhar. 1993. Immunocytochemical and biochemical characterization of the Heymann nephritis antigenic complex in rat L2 yolk sac cells. *Am. J. Pathol.* 143:1423-1435.
4. Orlando, R. A., D. Kerjaschki, H. Kurihara, D. Biemesderfer and M. G. Farquhar. 1992. Gp330 associates with a 44 kDa protein in the rat kidney to form the Heymann nephritis antigenic complex. *Proc. Natl. Acad. Sci. U.S.A.* 89:6698-6702.
5. Farquhar, M. G., D. Kerjaschki, M. Lundstrom and R. A. Orlando. 1994. Gp330 and RAP: The Heymann nephritis antigenic complex. *Ann. N.Y. Acad. Sci.* 737:96-113.
6. Kancha, R. K., M. E. Stearns and M. M. Hussain. 1994. Decreased expression of the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor in invasive cell clones derived from human prostate and breast tumor cells. *Oncology Res.* 6:365-372.
7. Orlando, R. A. and M. G. Farquhar. 1994. Functional domains of the receptor-associated protein (RAP). *Proc. Natl. Acad. Sci. USA.* 91:3161-3165.
8. Orlando, R. A. and M. G. Farquhar. 1993. Identification of a cell line that expresses a cell surface and a soluble form of the gp330/RAP Heymann nephritis antigenic complex (HNAC). *Proc. Natl. Acad. Sci.* 90:4082-4086.
9. Czekay, R.-P., R. A. Orlando, L. Woodward, E. D. Adamson and M. G. Farquhar. 1995. The expression of megalin and LRP diverges in differentiating F9 embryonal carcinoma cells. *J. Cell Sci.* 108:1433-1441.
10. Stampfer, M. R. and J. C. Bartley. 1985. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benso[a]pyrene. *Proc. Natl. Acad. Sci. USA.* 82:2394-2398.
11. Biemesderfer, D., G. Dekan, P. Aronson and M. G. Farquhar. 1993. Biosynthesis of the gp330-44 kDa Heymann nephritis antigenic complex: Assembly takes place in the ER. *Amer. J Physiol.* 264:F1011-F1020.
12. Tokuyasu, K. T. 1986. Application of cryoultramicrotomy to immunocytochemistry. *J. Micros. (Oxf).* 143:139-149.
13. Tokuyasu, K. T. 1989. Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem. J.* 21:163-171.
14. Hobman, T. C., L. Woodward and M. G. Farquhar. 1995. Targeting of a heterodimeric membrane protein complex to the Golgi: Rubella virus E2 glycoprotein contains a transmembrane Golgi retention signal. *Mol. Biol. Cell.* 6:7-20.

15. Hendricks, L., M. McCaffery, G. E. Palade and M. G. Farquhar. 1993. Disruption of endoplasmic reticulum to Golgi transport leads to the accumulation of large aggregates containing β-COP in pancreatic acinar cells. *Mol. Biol. Cell.* 4:413-424.
16. Wilson, B. S., C. Nuoffer, J. L. Meinkoth, M. McCaffery, J. R. Feramisco, W. E. Balch and M. G. Farquhar. 1994. A rab 1 mutant affecting guanine nucleotide exchange promotes disassembly of the Golgi apparatus. *J. Cell Biol.* 125:557-571.
17. Farquhar, M. G., L. H. Hendricks, T. Noda and A. Velasco. 1992. Contributions of enzyme cytochemistry and immunocytochemistry to our understanding of the organization and function of the Golgi apparatus. *In:* Electron Microscopic Cytochemistry and Immunocytochemistry in Biomedicine. K. Ogawa and T. Barka, ed. Boca Raton, FL: CRC Press, pp. 441-479.
18. McCaffery, M. and M. G. Farquhar. 1995. Localization of GTPases (GTP-binding proteins) by indirect immunofluorescence and immunoelectron microscopy. *Meth. Enzym.* 257:259-279.
19. Foekens, J. A., M. Schmitt, W. L. J. van Putten, H. A. Peters, M. D. Kramer, F. Jänicke and J. G. M. Klijn. 1994. Plasminogen activator inhibitor-1 and prognosis in primary breast cancer. *J. Clin. Oncol.* 12:1648-1658.
20. Grøndahl-Hansen, J., I. J. Christensen, C. Rosenquist, N. Brünner, H. T. Mouridsen, K. Danø and M. Blichert-Toft. 1993. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extract of breast carcinomas are associated with poor prognosis. *Cancer Res.* 53:2513-2521.
21. Jänicke, F., M. Schmitt, L. Pache, K. Ulm, N. Harbeck, J. Höfler and H. Graeff. 1993. Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. *Breast Cancer Res. Treat.* 24:195-208.
22. Jankun, J., H. W. Merrick and P. J. Goldblatt. 1993. Expression and localization of elements of the plasminogen activation system in benign breast disease and breast cancers. *J. Cell. Biochem.* 53:135-144.
23. Reilly, D., L. Christensen, M. Duch, N. Nolan, M. J. Duffy and P. A. Andreasen. 1992. Type-1 plasminogen activator inhibitor in human breast carcinomas. *Int. J. Cancer.* 50:208-214.
24. Schmitt, M., L. Goretzki, F. Jänicke, J. Calvete, M. Eulitz, H. Kobayashi, N. Chucholowski and H. Graeff. 1991. Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer. *Biomed. Biochim. Acta.* 50:731-741.
25. Damjanovich, L., C. Turzó and R. Adány. 1994. Factors involved in the plasminogen activation system in human breast tumours. *Thrombosis and Haemostasis.* 71:684-691.
26. Bastholm, L., M. H. Nielsen, J. De Mey, K. Danø, N. Brünner, G. Høyer-Hansen, E. Rønne and F. Elling. 1994. Confocal fluorescence microscopy of urokinase plasminogen activator receptor and cathepsin D in human MDA-MB-231 breast cancer cells migrating in reconstituted basement membrane. *Biotech. Histochem.* 69:61-67.
27. Jänicke, F., M. Schmitt and H. Graeff. 1991. Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. *Sem. Throm. Hemo.* 17:303-312.
28. Bianchi, E., R. L. Cohen, A. T. Thor, R. F. Todd, I. F. Mizukami, D. A. Lawrence, B. M. Ljung, M. A. Shuman and H. S. Smith. 1994. The urokinase receptor is

expressed in invasive breast cancer but not in normal breast tissue. *Cancer Res.* 54:861-866.

29. Zhang, S., M. Laurent, R. Lopez-Alemany, A. Mazar, J. Henkin, E. Ronne and P. Burtin. 1993. Comparative localization of receptors for plasmin and for urokinase on MCF7 cells. *Exp. Cell Res.* 207:290-299.

30. Williams, S. E., M. Z. Kounnas, K. M. Argraves, W. S. Argraves and D. K. Strickland. 1994. The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein and the receptor-associated protein. *Ann. N.Y. Acad. Sci.* 737:1-13.

31. Gliemann, J., A. Nykjær, C. M. Petersen, K. E. Jørgensen, M. Nielsen, P. A. Andreasen, E. I. Christensen, A. Lookene, G. Olivecrona and S. K. Moestrup. 1994. The multiligand α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP). *Ann. N.Y. Acad. Sci.* 737:20-38.

32. Moestrup, S. K., E. I. Christensen, S. Nielsen, K. E. Jørgensen, S. E. Bjørn, H. Røigaard and J. Gliemann. 1994. Binding and endocytosis of proteins mediated by epithelial gp330. *Ann. N.Y. Acad. Sci.* 737:124-137.

33. Nykjær, A., L. Kjøller, R. L. Cohen, D. A. Lawrence, B. A. Garni-Wagner, R. F. Todd, A.-J. van Zonneveld, J. Gliemann and P. A. Andreasen. 1994. Regions involved in binding of urokinase-type-1 inhibitor complex and pro-urokinase to the endocytic α_2-macroglobulin receptor/low density lipoprotein receptor-related protein: evidence that the urokinase receptor protects pro-urokinase against binding to the endocytic receptor. *J. Biol. Chem.* 269:25668-25676.

34. Czekay, R. P., R. A. Orlando, L. Woodward, M. Lundstrom and M. G. Farquhar. 1997. Endocytic trafficking of megalin/RAP complexes: dissociation of the complexes in late endosomes. *Molec. Biol. Cell.* 8:517-532.

ANIMAL USE REPORTING

Activity Name & Address: University of California, San Diego 9500 Gilman Drive, La Jolla, CA 92093-0651

Contract number: DAMD 96-1-6317

Animal Type Genus/Species	Animals Purchased or Bred	Animals Used	USDA Pain Column C	USDA Pain Column D	USDA Pain Column E	AAALAC Accreditation
Rabbit (New Zealand White)	2	2	Category A: Minimal Discomfort 2	Ø	Ø	UCSD Accredited 2/9/96-2/8/99 Rabbit Use Approved6/18/97

USAMRAA FORM 52-R (NOVEMBER 1994)

TABLE I

Available Antibodies

Antibodies	Type	Designation	Assays		
			IF	IB	IP
anti-human LRP (recognizes the cytoplasmic tail)	PAb	anti-LRP_{ct} (human)	+	+	+
anti-human LRP	PAb	anti-LRP (human)	+	+	+
anti-rat megalin (recognizes the cytoplasmic tail)	PAb	anti-$megalin_{ct}$ (human)	+	+	+
anti-rat megalin	PAb	anti-megalin (rat)	+	+/-	+
anti-rat megalin (against ligand-binding domains I or II)	PAb	anti-$megalin_{LBD\ I,II}$ (rat)	+	+	+
anti-human megalin	PAb	anti-megalin (human)	+	+	+
anti-human uPAR	PAb	anti-uPAR (human)	+	+	+
anti-human uPAR (American Diagnostica)	MAb	3936	+	nd	nd
anti-human uPAR (American Diagnostica)	MAb	3937	+	nd	nd
anti-human uPA (B chain) (American Diagnostica)	MAb	394	+	+	+
anti-rat RAP	PAb	anti-RAP (rat)	-	+	+
anti-human RAP	PAb	anti-RAP (human)	+	+	+

+/- = of limited use
+ = useful in the indicated assay
nd = not done
PAb = polyclonal antibody
MAb = monoclonal antibody

IF = immunofluorescence
IB = immunoblotting
IP = immunoprecipitation

TABLE II

Expression of LRP, megalin, and RAP in normal and malignant breast cancer cells

	LRP			Megalin		RAP	
Cell line	**IF (perm)**	**IF (surface)**	**IB**	**IF**	**IP**	**IF**	**IB**
184-B5	+++	+	+	-	nd	+++	+
MCF-7	+++	nd	+/-	+	nd	+++	+++
Hs578T	+++	++	+	+++	++	+++	+++
MDA-MB-231	+++	++	+++	++	nd	+++	+++

perm. = permeabilized
surface = surface labeling
IF = immunofluorescence
IB = immunoblot
IP = immunoprecipitation

\- = not detected
+/- = very weak
\+ = weak
++ = moderate
+++ = strong

TABLE III

Expression of uPAR, uPA, and PAI-1 in normal and breast cancer cells

	uPAR			uPA	PAI-1
Cell line	IF (perm)	IF (surface)	IB	IF	IF
184-B5	+++	+	+	+	-
MCF-7	+++	+	+++	-	-
Hs578T	+++	+++	+++	+++	++
MDA-MB-231	+++	+++	+++	+++	+++

perm. = permeabilized
surface = surface labeling
IF = immunofluorescence
IB = immunoblot

\- = not detected
\+ = weak
++ = moderate
+++ = strong

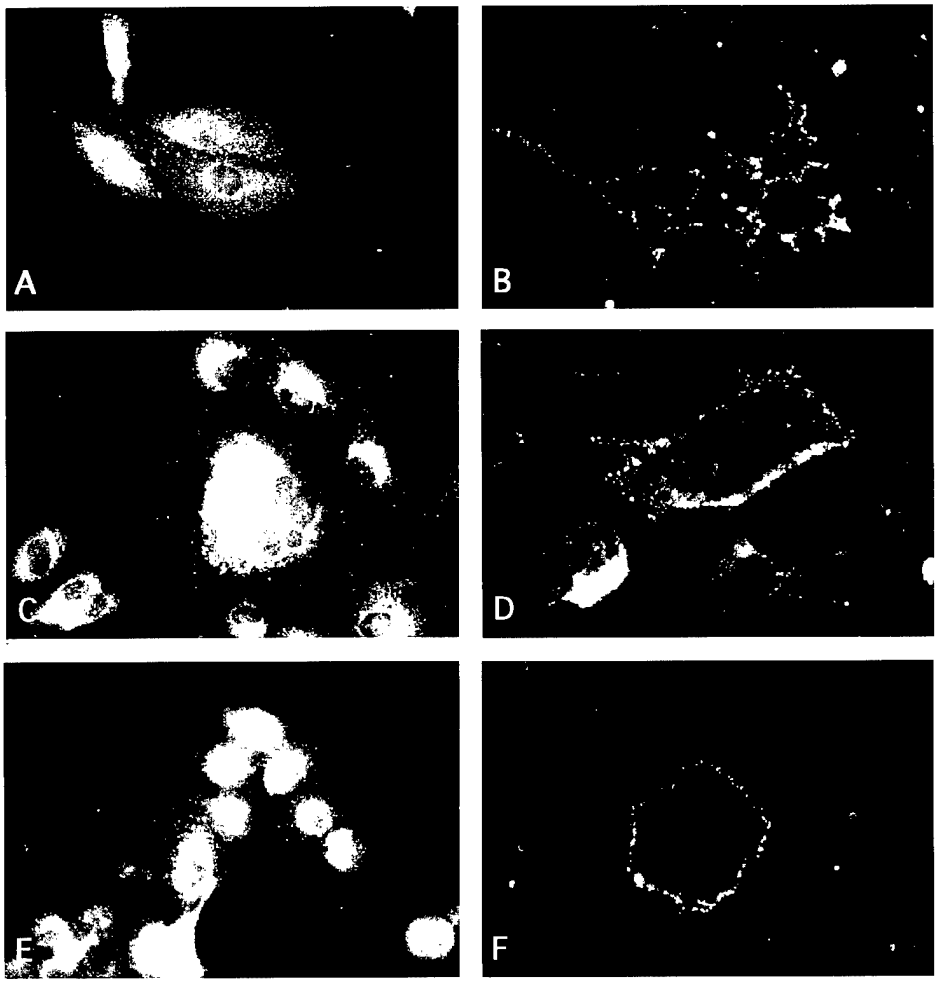

Figure 1: By immunofluorescence LRP is detected in breast cancer cell lines as well as in a normal mammary epithelial cell line

A. MDA-MB-231 cells: permeabilized
B. MDA-MB-231 cells: non-permeabilized (surface labeling)
C. Hs578T cells: permeabilized
D. Hs578T cells: non-permeabilized (surface labeling)
E. 184-B5 cells: permeabilized
F. 184-B5 cells: non-permeabilized (surface labeling)

For surface labeling cells were incubated at 4°C with purified anti-LRP (human) (24 μg/ml) without prior detergent permeabilization followed by incubation with FITC-donkey anti-rabbit F(ab')2, fixed in 2% paraformaldehyde for 1 hr, and examined by epifluorescence. For detection of intracellular LRP, cells were fixed in 2% paraformaldehyde and permeabilized with 10 mM CHAPS or 0.05% saponin followed by incubation with affinity purified anti-LRPct (human) and the same secondary antibody as for non-permeabilized cells.

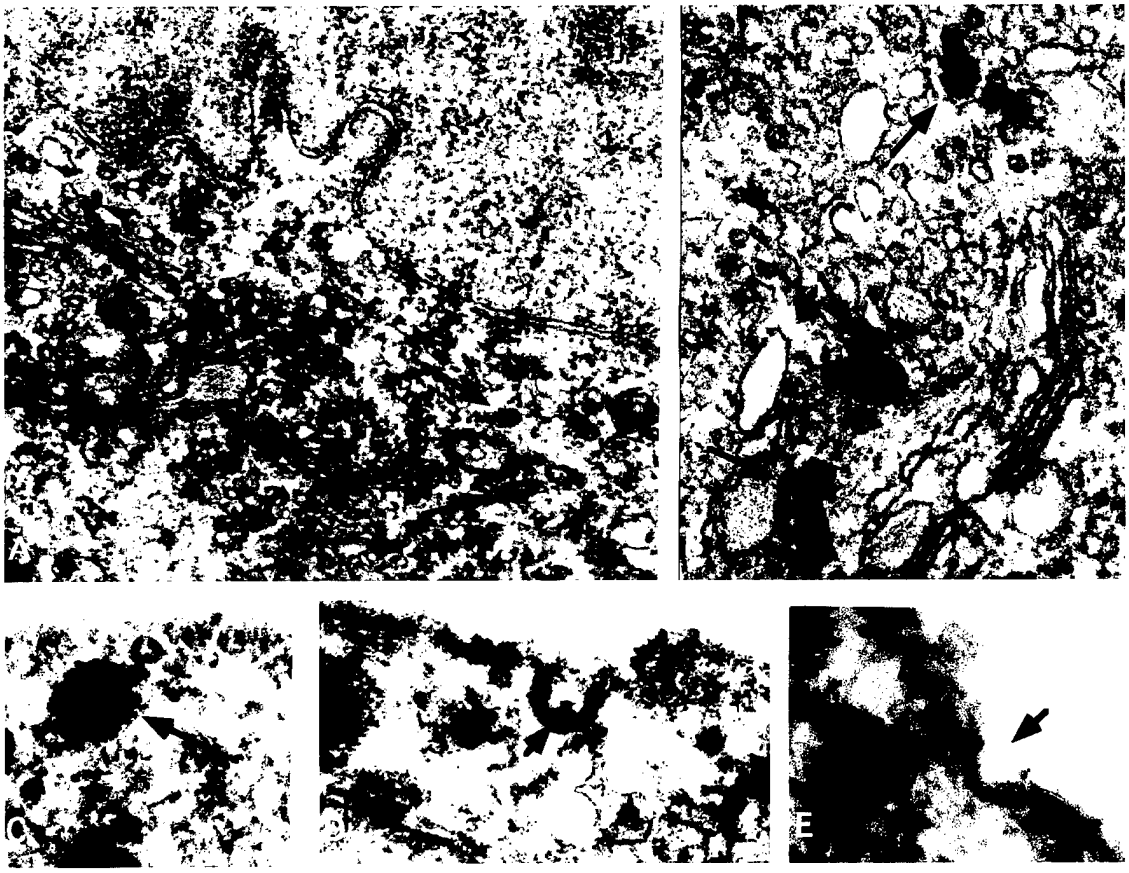

Figure 2: By immunoelectron microscopy LRP is found in clathrin-coated pits at the cell surface and in Golgi elements of Hs578T cells

A and B. Immunoperoxidase localization of LRP in Golgi cisternae and associated vesicles (arrows).
C and D. Immunoperoxidase localization of LRP in a coated vesicle near the cell membrane (arrow) and coated pits on the cell membrane, respectively.
E. Immunogold localization of LRP in a coated pit (arrow) on the cell surface.

For the immunoperoxidase procedure, cells were incubated with anti-LRP (human) antibodies followed by a sheep anti-rabbit Fab-peroxidase conjugate (A-D) or a goat anti-rabbit gold (5 nm) conjugate (E). Methods for the preparation of cells and processing for immunoperoxidase and immunogold labeling are given in the Body of the Progress Report.

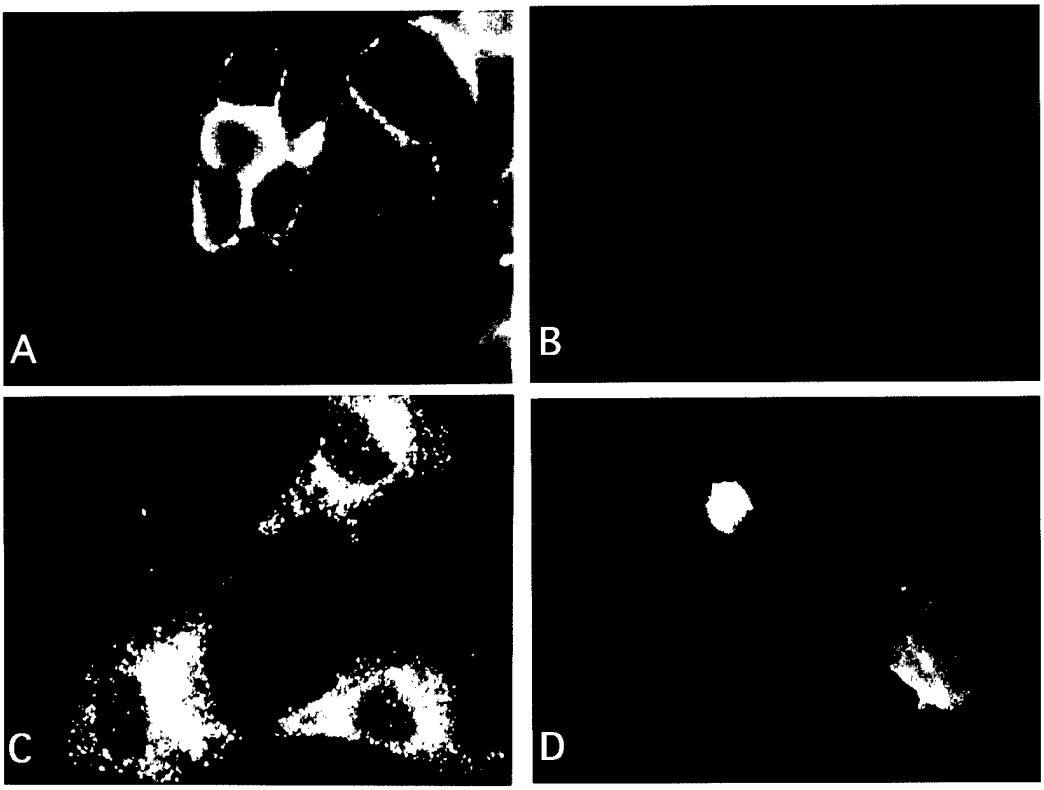

Figure 3. Coated pit localization of megalin in breast cancer cells. Immunofluorescent labeling of megalin shows a punctate staining on the cell surface, consistent with a coated pit localization.

(A) JEG3 cells, positive control for the anti-megalin (human) antibody.

(B) the normal mammary cell line 184-B5 does not express megalin.

(C) and (D) Hs578T cells stained with the anti-megalin$_{ct}$ (rat) or with the anti-megalin (human) antibodies, respectively. Note the localization of megalin at the leading edge in Hs578T cells (D).

Immunofluorescence staining was performed as described in Figure 1.

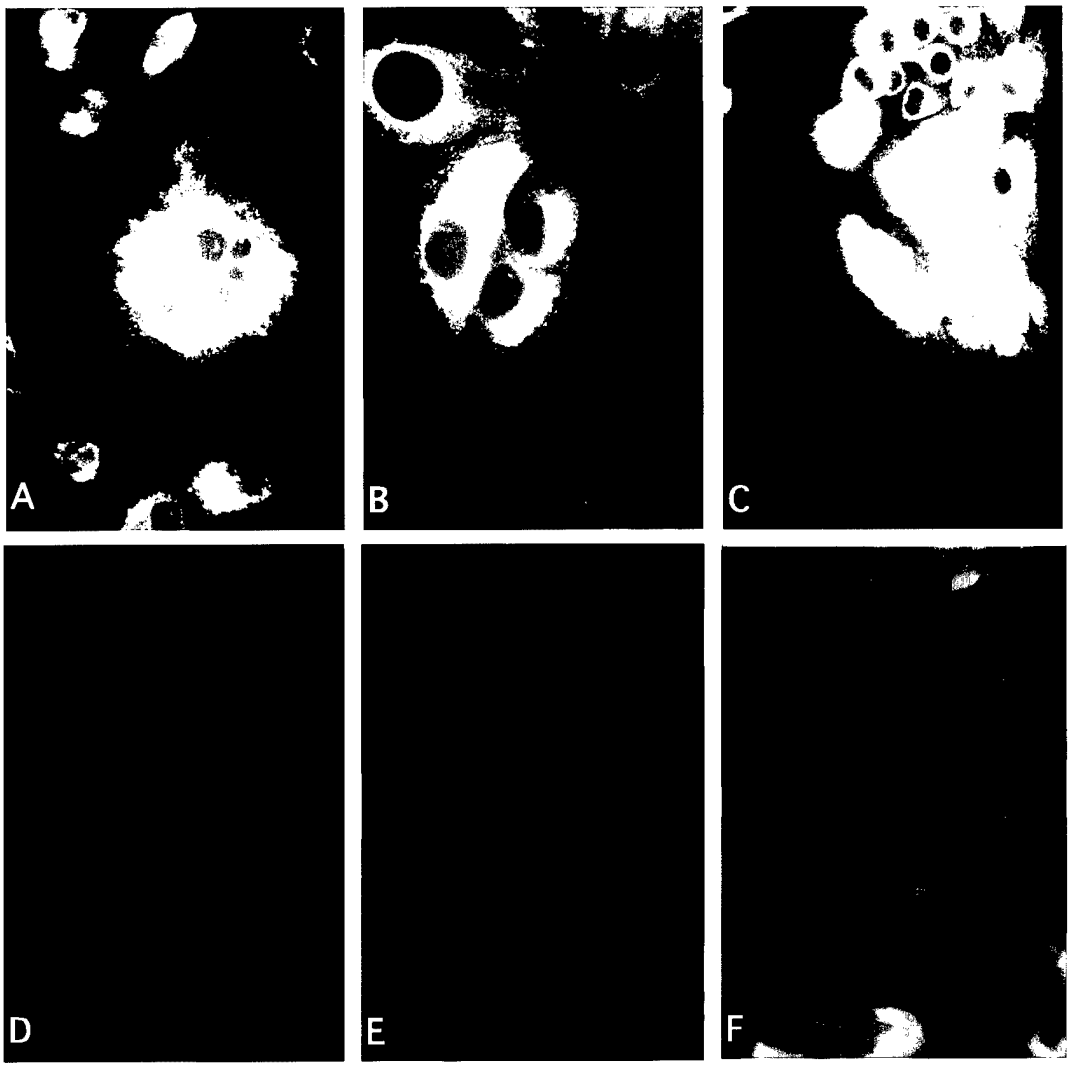

Figure 4: RAP is concentrated in the ER of normal and tumor-derived cells

A. MDA-MB-231 cells stained for RAP
B. MCF-7 cells stained for RAP
C. 184-B5 cells stained for RAP
D and E. Hs578T cells stained for RAP (D) and the ER marker, protein disulfide isomerase (PDI) (E)
F. Merged image of D and E showing overlap of RAP with PDI

Single labeling was done as described in Figure 1. For double labeling paraformaldehyde fixed cells were incubated in rabbit polyclonal anti-RAP (human) and mouse monoclonal anti-PDI (StressGen) followed by incubation in FITC-anti-mouse Fab'2 and TRITC-anti-rabbit F(ab)'2.

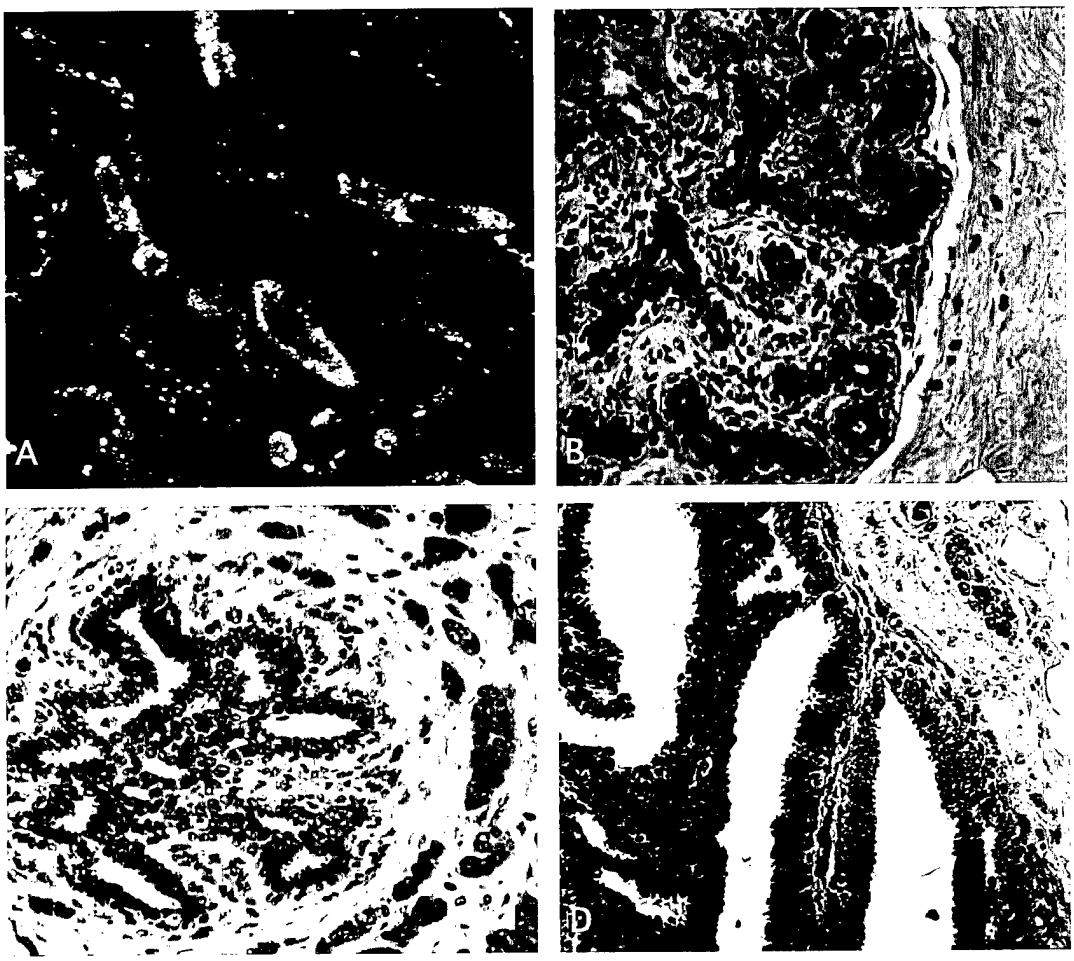

Figure 5. LRP can be detected in MDA-MB-231 tumors grown in nude mice as well as in primary ductal breast cancer.

A. MDA-MB-231 cells, derived from tumor cells grown in nude mice.
B-D. Immunoperoxidase staining of paraffin sections of an infiltrating ductal breast carcinoma. Well differentiated epithelial tissue is detectable at tumor margins (B).

For immunofluorescence (A), tumors from nude mice injected with MDA-MB-231 cells were removed, cryosections were prepared and incubated with anti-LRPct (human) followed by FITC-donkey anti-rabbit F(ab')2. For immunoperoxidase (B-D), 5 μm sections of human breast cancer biopsy tissue were processed as described in the Body of the Progress Report using anti-LRP (human) antiserum.

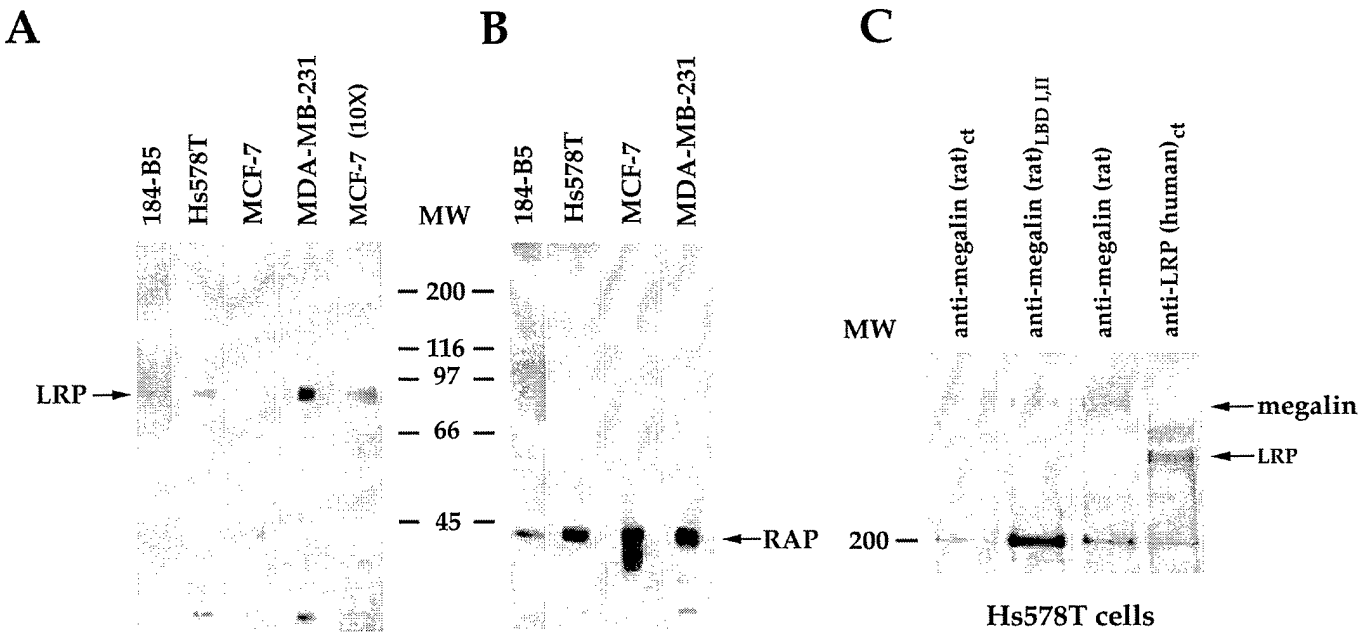

Figure 6: Expression of scavenger receptors and RAP in normal and tumor-derived mammary epithelial cell lines. For immunoblotting (A, B) proteins were extracted in 10 mM CHAPS, equal amounts of protein were separated by SDS-PAGE, and transferred to PVDF membranes for immunoblotting with anti-LRPct (human) and anti-RAP (human) antibodies.

LRP (A): Highest amounts of LRP were expressed in estrogen-insensitive tumor-derived cell lines MDB-MB-231 and Hs578T, whereas in the estrogen-sensitive cell line, MCF-7, LRP expression could only be demonstrated when the amount of protein electrophoresed was increased 10-fold (MCF-7 (10X)).

RAP (B): All tumor-derived cell lines expressed similar amounts of RAP and expression levels were slightly higher than in the normal cell line, 184-B5.

Megalin (C): Hs578T cells were metabolically labeled with 35S-methionine/cysteine and proteins were extracted in 10 mM CHAPS. Aliquots were incubated with anti-megalin (rat) antibodies bound to protein A-agarose beads. Antibody-bound proteins were processed for and separated by SDS-PAGE. Megalin was detected in the estrogen-insensitive cell line, Hs578T, with three anti-megalin (rat) antibodies.

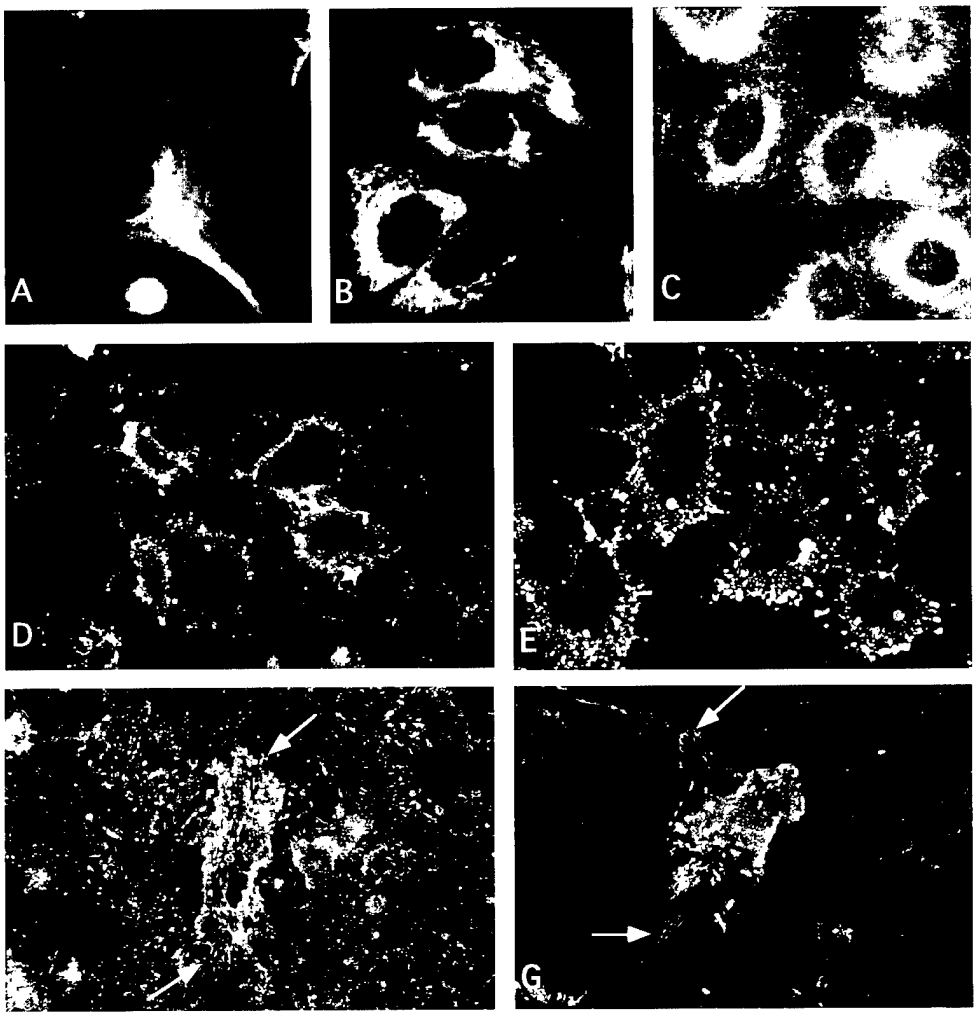

Figure 7. Intracellular and surface distribution of uPAR in normal and tumor-derived mammary epithelial cell lines

A. Hs578T cells: permeabilized.
B. MCF-7 cells: permeabilized.
C. 184-B5 cells: permeabilized.
D and F. MDA-MB-231 cells: non permeabilized (surface labeling).
E and G. Hs578T cells: non permeabilized (surface labeling).

For immunofluorescence labeling of cells permeabilized with CHAPS, the polyclonal antibody against human uPAR was used. For surface labeling, we used either MAb #3936 (20 µg/ml) (D, E) or MAb #3937 (10 µg/ml) (F,G). Note the focal adhesion - like staining pattern (arrows) in surface labeled MDA-MB-231 and Hs578T cells.

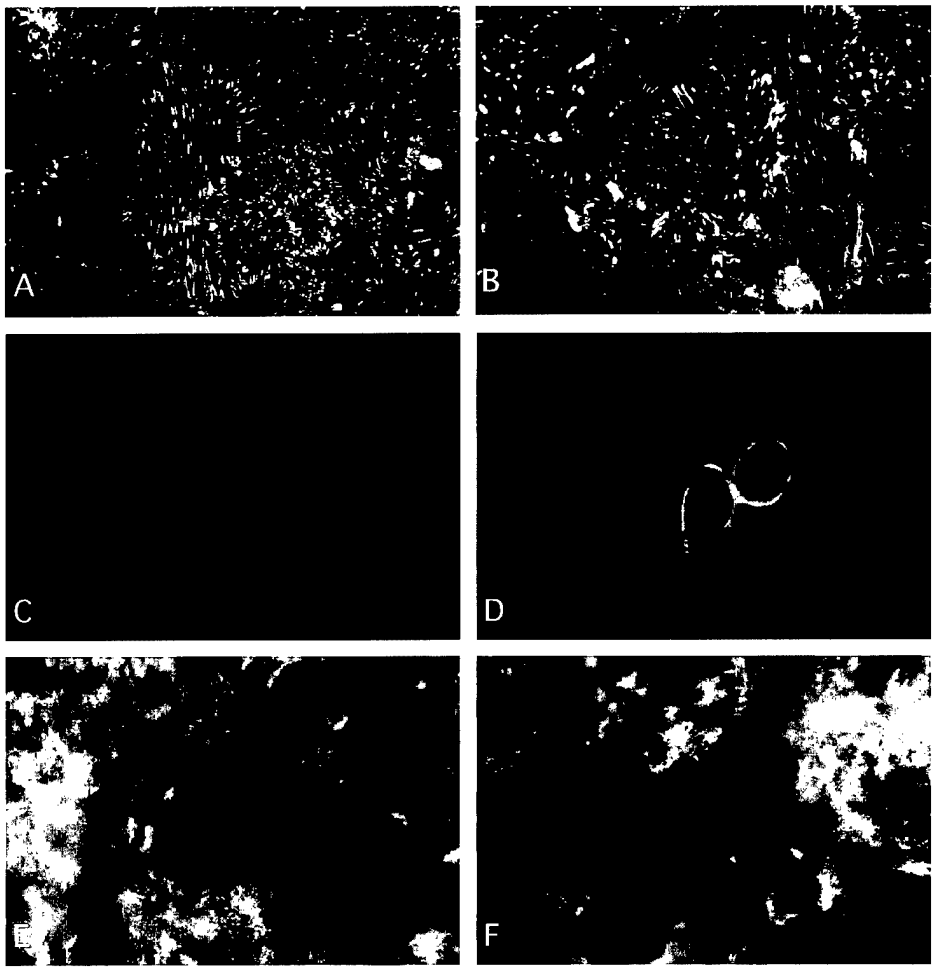

Figure 8. Distribution of uPA and PAI-1 in normal and tumor-derived epithelial cell lines

A. MDA-MB-231 cells stained for uPA
B. Hs578T cells stained for uPA
C. MCF-7 cells stained for uPA
D. 184-B5 cells stained for uPA
E. MDA-MB-231 stained for PAI-1
F. Hs578T cells stained for PAI-1

Non-permeabilized cells were either stained with MAb #394 against human uPA (20 μg/ml) (A-D) or with MAb anti-human PAI-1 (10 μg/ml) (E, F) as described in Fig.1. Diffuse staining patterns observed for MDA-MB-231 and Hs578T cells using anti-PAI-1 MAb are due to the localization of PAI-1 within the ECM.

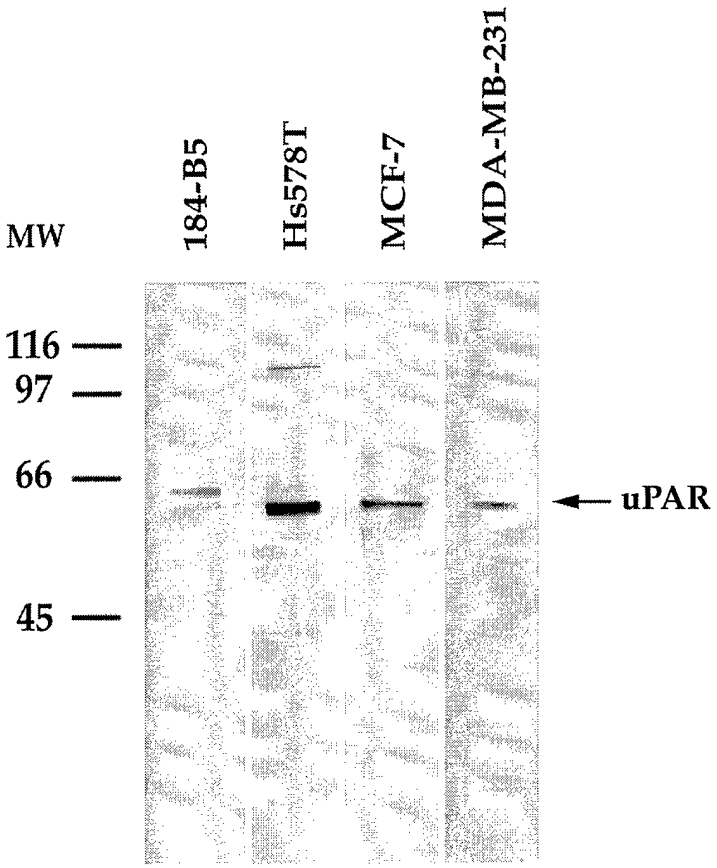

Figure 9: By immunoblotting uPAR is expressed in normal and tumor-derived mammary epithelial cell lines. Protein extracts were prepared and processed for immunoblotting (as in Figure 6) with anti-uPAR (human) antibodies. All tumor-derived cell lines express uPAR with no significant differences in the expression levels, but all breast cancer cell lines express more uPAR than does the normal mammary cell line, 184-B5.

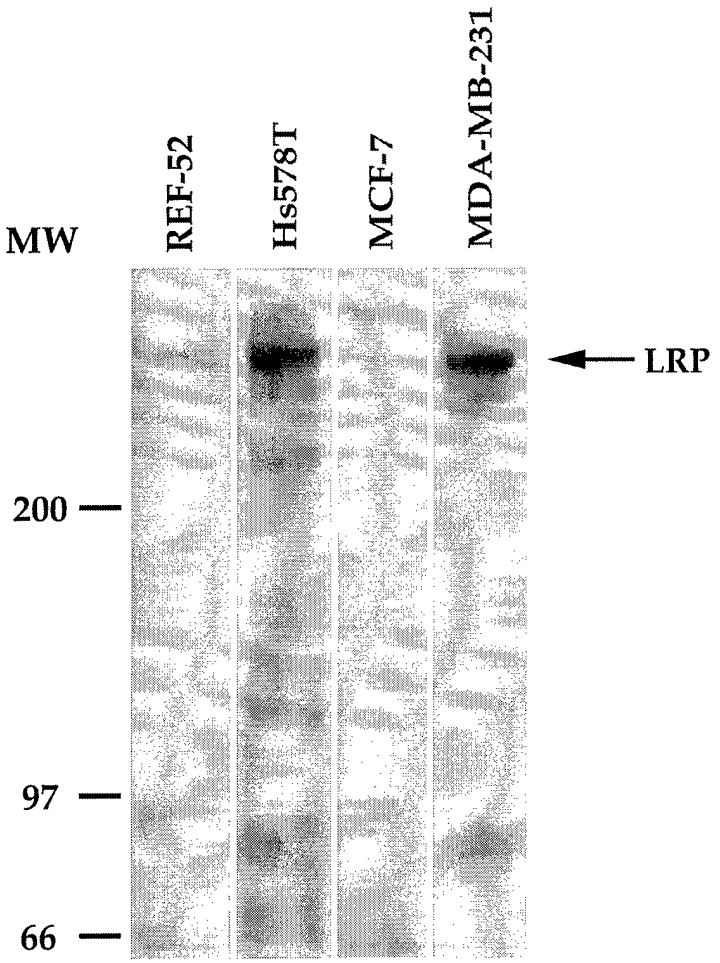

Figure 10: Cell surface expression of LRP in tumor-derived mammary epithelial cell lines. Cells were radiolabeled by lactoperoxidase-mediated cell surface iodination. Cells were lysed in 10 mM CHAPS and radiolabeled proteins from each cell line were immunoprecipitated using anti-LRP (human) antibodies bound to protein A-agarose beads. Precipitated proteins were processed and separated by SDS-PAGE. Expression of LRP is much greater in estrogen-insensitive MDA-MB-231 and Hs578T cells than in estrogen-sensitive MCF-7 cells where the receptor was barely detectable. REF-52 (rat embryonal fibroblasts), used as a positive control in this experiment, also expressed much lower levels of LRP than the two estrogen-insensitive breast cancer cell lines.

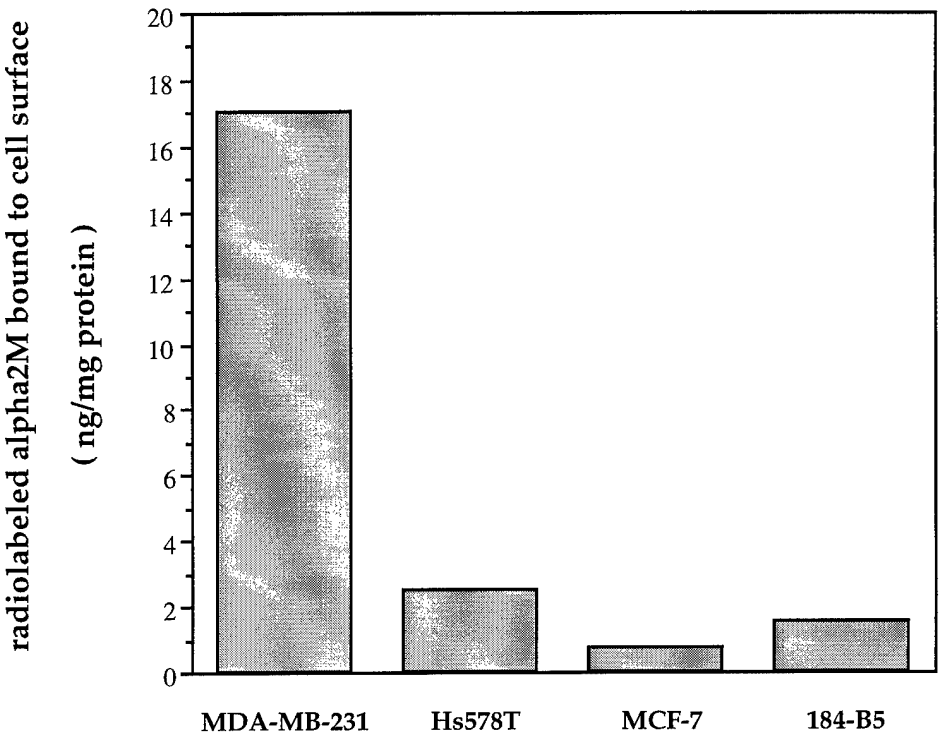

Figure 11: α2macroglobulin (α2M) binding capacities on normal and tumor-derived mammary epithelial cell lines. 125I-α2M was bound to cells at 4°C for 3 hr. Cell associated radioactivity was quantitated by gamma counting and normalized to total cellular protein. Binding capacity of LRP in MDA-MB-231 cells was 6-17X greater than in the normal mammary cell line or breast cancer cell lines.

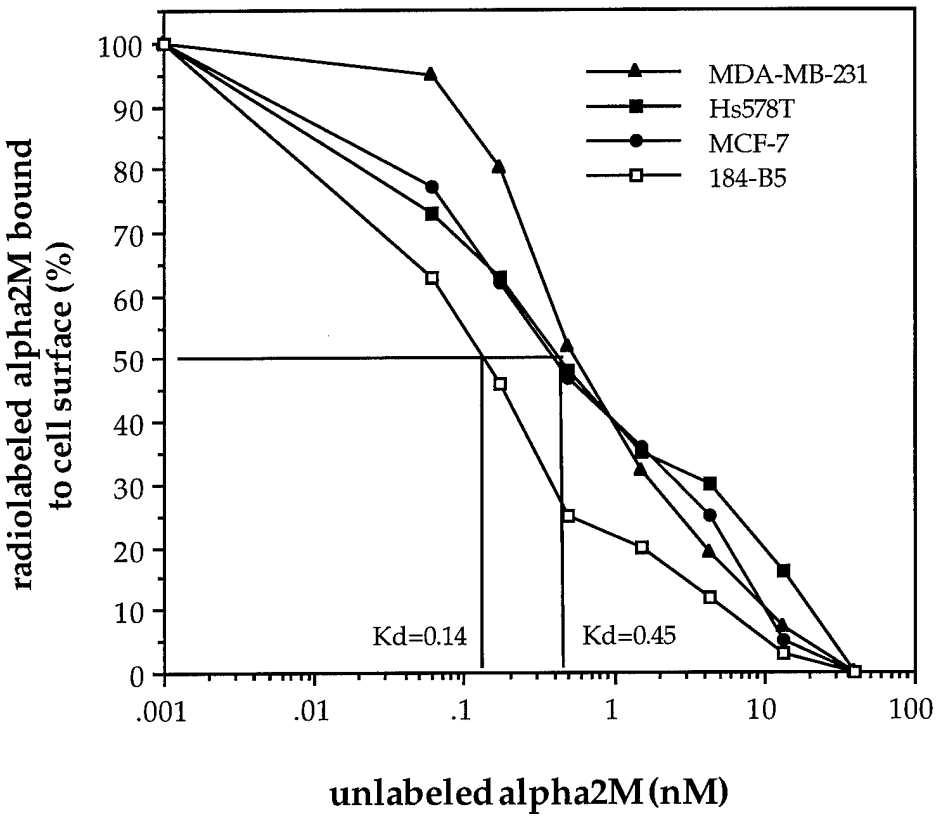

Figure 12: α2macroglobulin binding affinities to LRP in normal and tumor-derived mammary epithelial cell lines. Cells were incubated at 4°C with radiolabeled 125I-α2M (2 nM) in the presence of increasing amounts of unlabeled α2M (0-40 nM). The concentration of unlabeled α2M which resulted in 50% inhibition of 125I-α2M binding represents the binding affinity (Kd). All three breast cancer cell lines demonstrated a slightly lower affinity for α2M binding to LRP (Kd=0.45 nM) than the normal cells (Kd=0.14 nM).

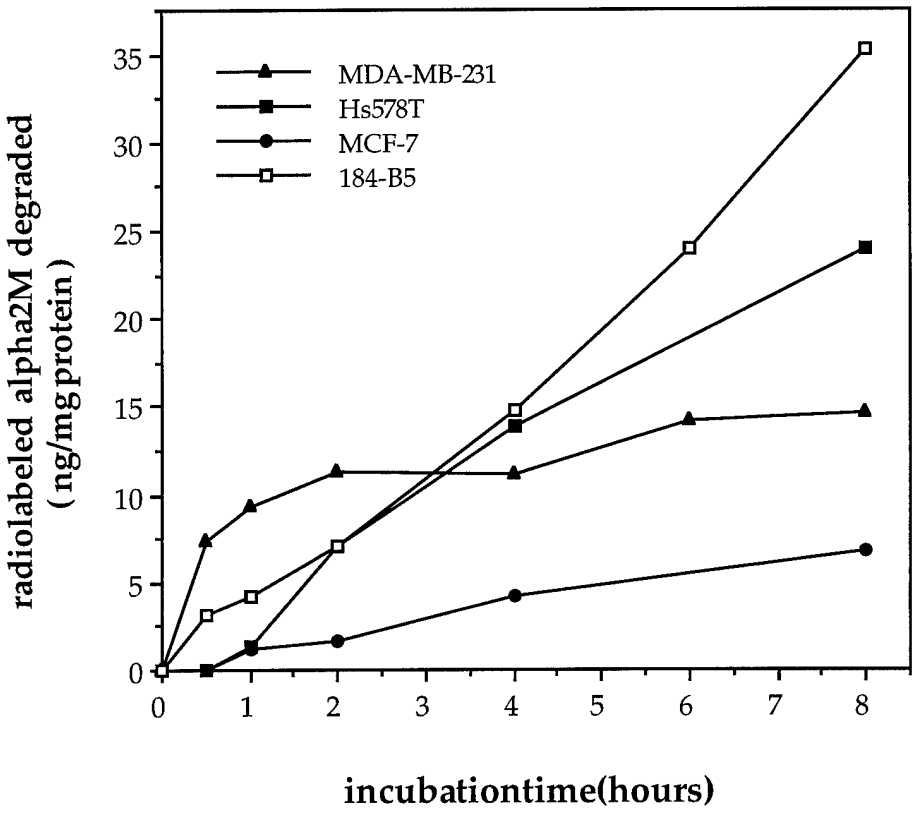

Figure 13: LRP-mediated internalization and degradation of 125I-α2M in normal and tumor-derived mammary epithelial cell lines. Cells were incubated with radiolabeled α2M (2 nM) at 37°C and the media sampled at various times, adjusted to 10% TCA, and non-precipitable material was measured by gamma counting. Hs578T and normal epithelial cells (184-B5) degraded 125I-α2M in a linear, time-dependent manner. MCF-7 cells showed little degradation of the ligand. Interestingly, MDA-MB-231 cells demonstrated rapid uptake and degradation of 125I-α2M followed by very little degradation over the remaining 6 hr time course.